

4 Chapter 4: General Discussion

4.1 Introduction

Cervical cancer is rated the second most common malignant tumour in women worldwide. South Africa has the highest incidence of cervical cancer globally (<http://www.cansa.org.za/Research/registry> 1997_cervix.asp). It commonly affects African and Coloured South African women. Cervical cancer is linked to a sexually transmitted disease caused by some strains of human papillomavirus. It is more common in women who have multiple sexual partners or who began to have intercourse before age 18. Cervical cancer has also been associated with cigarette smoking and a diet deficient in folic acid. Cervical cancer is classified as either mild (Cervical intraepithelial neoplasia, CIN I), moderate (CIN II) or severe (CIN III) or carcinoma-in-situ (Fletcher, 1993).

A normal cervix is covered by a layer of squamous cells. There are many of these cell layers before the first flattened layer of cells with nuclei. At the lower portion of the cell layers the cells are round and younger. As the cells mature they rise to the surface and become flat. The squamous cells are separated from the underlying structures by a basement membrane. In mild dysplasia only a few cells are abnormal. In moderate dysplasia the abnormal cells involve about one-half of the thickness of the surface lining of the cervix. In carcinoma-in-situ the entire thickness of abnormal cells have not yet spread below the surface or basement membrane. With invasive cancer, the cells are not only abnormal throughout the entire thickness from the top to the basement membrane, but they invade the basement membrane.

This study tried to elucidate the possible role of DWNN in human cervical cancer and apoptosis and also established the expression pattern and expression levels of DWNN in human cervical cancer. This was accomplished by extracting RNA which was reverse transcribed, and then PCR amplification was done using gene specific primers. The PCR product was further cloned into pGEM T Easy then linearized to in vitro transcribe RNA probes that were used for *in situ* hybridization. The synthesized anti-human DWNN antibodies against DWNN-13 kDa and DWNN-200 kDa were used for localizing DWNN proteins. To elucidate its role in apoptosis, TUNEL procedure performed was to correlate the apoptosis levels with the DWNN expression; this was further confirmed by determining the expression pattern of Bcl-2 in this disease. To assess the proliferative status of cervical cancer samples, a proliferation assay was done using ki67 antigen.

DWNN does not have a distinct function yet. Studies have been carried out on this gene and its protein product, including cellular localization studies in various carcinomas, and possible cellular mechanisms that control the apoptotic cascade. Molecular structural studies were done to ascertain the function of the DWNN proteins (Mbita, unpublished data- MSc thesis).

4.2 DWNN expression and localization in cervical cancer.

4.2.1 *In situ* hybridization

In situ hybridization is one of the techniques that form a foundation of molecular developmental biology, with which patterns of gene expression to locate the chromosomal location of a specific RNA probe are undertaken. In colometric *in situ* hybridization, the probe is labelled with digoxigenin and detected using substrate NBT/BCIP and viewed under the light microscope. Fluorescent *in situ* hybridization was done to achieve fluorescent labelling of mRNA with high sensitivity and higher resolution than the colometric *in situ* hybridization. This technique was undertaken in this study to localize DWNN mRNA transcripts and to determine DWNN levels in cervical cancer tissue sections and normal tissue sections. In a previous study (Ko *et al.*, 2002), *in situ* hybridization was used to determine expression of human secreted frizzled related protein (hsFRP) in cervical cancer tissues and cancer cell lines and compared with normal cervical tissues.

To confirm the specificity of labelling the probes for all the DWNN transcripts; 5' 1.1 kb, 3' 1.1 kb, 3' 6.1 kb and exon 16 were synthesized.

The 5' 1.1 kb mRNA probe was up-regulated in malignant disease in contrast to normal cervical tissue. The probe was localized both in the cytoplasm and nuclei. This was highly expressed in moderately differentiated squamous cell carcinoma and the surrounding stroma, but down-regulated in well-differentiated squamous cell carcinoma. Since 5'1.1 kb mRNA is able to detect all the three transcripts (1.1, 6.1 kb (spliced

variant) and 6.1 kb (unspliced variant), therefore overexpression was expected. Nuclear staining indicates that DWNN gene was transcribed for a reason, and the cytoplasmic cellular localization suggests that the translated protein was in high demand for a specific function. It was highly expressed in moderately differentiated cells because it may be combating disease. Apoptosis regulates tumour progression by counteracting cell proliferation by cell death. Therefore, up-regulation of DWNN supports its involvement in apoptosis.

Cellular localization of DWNN 3' 1.1 kb mRNA probe was found in the cytoplasm and nucleus. It was up-regulated in moderately differentiated squamous cell carcinoma and the surrounding area, but down-regulated in normal tissue sections and well-differentiated squamous cell carcinoma. This detects only the 1.1 kb transcript. Even though it was highly expressed as compared to the normal tissue, this was not as elevated as the 5' 1.1 kb. Its up-regulation indicates that the gene was highly needed for the apoptotic processes.

The 3' 6.1 kb mRNA probe detects the 6.1 kb transcript and this comes in two forms; the unspliced and spliced forms of exon 16. This was highly expressed in moderately differentiated squamous cell carcinoma and the surrounding stroma, whilst well differentiated squamous cell carcinoma showed low levels of expression. It was also localized in the cytoplasm and nucleus. Its up-regulation might be because the encoded protein is associated with apoptosis related domains, which indicates that DWNN plays a vital role in apoptosis.

Alternative splicing of exon 16 is found at the 6.1kb transcript. This exon was highly expressed in moderately differentiated squamous cell carcinoma and the surrounding stroma. Its up-regulation implies that the unspliced 6.1 transcript is highly expressed as compared to the spliced form. If there were low levels of exon 16 this would indicate that the spliced form is up-regulated. Lightcycler RT-PCR showed increased accumulation of 5' 1.1 kb transcript compared with the 3' 6.1 kb transcript in the cancerous cell line HT 29. The exon 16 transcript was also highly accumulated. These results correlated well with the *in situ* hybridization results.

Fluorescent *in situ* hybridization for all the above probes was undertaken to confirm the specificity of colorimetric *in situ* hybridization. All the above mentioned probes showed the same pattern of expression, but only nuclear localization was observed. Nuclear localization might demonstrate that novel DWNN is a nuclear protein. DWNN may function both in the cytoplasm and nucleus, although the biological significance of the nuclear DWNN protein we identified remains to be identified. As a nuclear protein DWNN may function to facilitate transcription or DNA replication. Alternatively, it may be performing a structural role throughout the progression of the cell cycle.

4.2.2 Immunocytochemistry

This is a technique that uses an antigen-antibody reaction coupled with a reaction that produces a chromogen, a coloured product, to identify specific components in tissues. To further investigate the potential role of DWNN in cervical cancer, we examined the expression of the DWNN protein product in cervical cancer and normal tissue sections.

The DWNN proteins were highly expressed in the cytoplasm and some nuclei of moderately differentiated carcinoma and well differentiated carcinoma. Its high levels of cytoplasmic staining were also observed in moderate and severe dysplasia. In the normal cervix, the DWNN immunoreactivity was restricted at the intermediate layer, but down-regulated in the other layers. The unlabelled intermediate layer suggests that oestrogen, which influences the amount of glycogen in this layer, might suppress DWNN expression. The keratinized cells also expressed the protein. This indicates the protein was expressed in high grade lesions which were already keratinized. The abnormal cancer cells showed high expression levels of DWNN 13kDa.

4.2.3 Image analysis

Labelling intensity of the DWNN protein was determined by image analysis. In order to obtain a unit that could be used across all the images, the total number of pixels that represent immunolabelling were calculated per area, so the unit that was used is pixels / μm^2 .

The error bars illustrating the differences in staining are shown in figure 3.33. For the statistical analysis; an analysis of variance by ANOVA 1 was conducted and the effect of staining was significant, $F=12$; $p=0.000$. The Turkey HSD procedure revealed that all pairwise differences among means were significant, $p < 0.05$. Kruskal-Wallis' test a nonparametric alternative to the one-way analysis of variance (ANOVA 1), supported the ANOVA results.

There were significantly elevated levels of DWNN protein at all grades of invasive carcinoma and those of dysplasia. The keratin pearls indicated high expression of the DWNN protein with a value of 35 pixels/ 100 μm^2 . The image analysis results correlated well with the light microscopy images.

DWNN was up-regulated in the more undifferentiated cervical cancer grades in contrast to normal tissue sections. DWNN up-regulation was directly proportional to the high apoptosis levels in moderately differentiated squamous cell carcinoma and the invaded stroma. Apoptosis is thought to be the hallmark in therapeutic strategies for cervical cancer, in which it eliminates the abnormal exponentially proliferating cells (Nair *et al.*, 1999). Apoptosis was identified in poorly differentiated, moderately differentiated squamous cell carcinoma and the surrounding stroma but low labelling in well differentiated squamous cell carcinoma and this is where the DWNN was up-regulated.

The significant correlation between high apoptosis levels and DWNN expression suggests that the apoptotic cascade is initiated and DWNN may be essential in this cascade.

This study showed elevated levels of DWNN at moderately differentiated carcinoma. It is highly expressed at the sites where p53 was found to be highly expressed, as reported in the studies by Nair *et al.*, 1999 and Grace *et al.*, 2003. DWNN followed the same expression pattern as p53. This suggests that DWNN may be a pro-apoptotic protein. Accumulation of high levels of DWNN supports the concept that DWNN-triggered apoptosis is mediated through a p53-dependent pathway.

4.3 Apoptosis detection using TUNEL.

Apoptosis is characterised by fragmentation of the genomic DNA. These many breakpoints were visualized with the terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling reaction (TUNEL). The enzyme terminal deoxynucleotidyl transferase adds biotinylated nucleotides to the broken DNA ends. This experiment was done to correlate the apoptosis levels with the expression levels of DWNN.

Suicide is the only option that can decide if life is or is not worth living. Usually a cell has three alternatives; to divide (mitosis), to specialize (differentiation) or to commit suicide (apoptosis). By maintaining a balance between these decisions, it guarantees tight regulation of cell numbers within organisms (Melino, 2001). During carcinogenesis many

cancer cells prevent their self-destruction by evading the normal apoptotic mechanisms, by acquiring genetic damage.

This experiment used an *in situ* technique that shows characteristic chromatic cleavage occurring during apoptosis, and it indicated a significant decrease as the cervical epithelium became increasingly neoplastic. The labelling of DNA fragments was low in a small fraction of some nests of well-differentiated squamous carcinoma cells, but labelling was strong in moderately differentiated squamous carcinoma cells. Invaded stroma showed high apoptosis levels. In contrast, Isaacson and colleagues (1996) reported apoptosis to increase with histological abnormalities but Shoji *et al* (1996) found a significant correlation with histological grading, with increased apoptosis with tumour cell invasion into the stroma.

The concept of HPV infections indicates the decreased ability of infected cells to undergo apoptosis as a result of inactivation of the cellular tumour suppressor gene products p53 and Rb by viral oncoproteins E6 and E7, respectively. Therefore, alterations in differentiation, proliferation and cell death were anticipated. These high levels of apoptosis were in accord with the high expression levels of DWNN, which suggests that DWNN may be involved in apoptosis.

4.4 Proliferation assay using Ki67 expression

Monoclonal antibody Ki67 identifies cells in G1, S, G2, and M phases of the cell cycle (Wong, 1994). In this study, Ki67 was used to characterize tumour cells in human cervical cancer tissue sections. There is a prompt cell turnover in the cervical epithelium. Normal cervical epithelial cells were estimated to have 4-5 days lifespan, but time of transit was considerably shortened for dysplastic cells (Zanotti *et al.*, 2003). Indeed, Ki67 expression showed a dramatic increase in the rate of proliferation with increasing severity of dysplastic epithelium, in accord with previous data. Wong (1994) reported that Ki67 showed immunoreactivity in a tumour cell population and it significantly correlated with mitotic activity of tumour cells. The rate of proliferation increased as the tumour progressed, and with consequence cell death must also increase to maintain tissue homeostasis.

High rates of proliferation suggested that cervical epithelial cells and invasive carcinoma cells entered terminal differentiation with tumour progression. The rate of proliferation was found to be indirectly proportional to DWNN expression. This finding indicated that Ki67 immunoreactivity provides an easily measurably index to study proliferating cell populations in cervical cancer.

4.5 Bcl-2 expression

The proto-oncogene Bcl-2 plays a vital role in apoptosis regulation (Nair *et al.*, 1999). The over-expression of Bcl-2 proteins can inhibit apoptosis and extend cell survival; therefore, it can play an important role in carcinogenesis. A decrease in Bcl-2 expression does not necessarily implicate an increase in apoptosis because other factors regulating apoptosis process may also be altered (Cheung *et al.*, 2002). The reliability of TUNEL procedure to distinguish only apoptotic cells has always been disputed, because phagocytosed and necrotic cells can also be stained by this technique. Because of these disputes, markers of apoptosis were investigated in order to validate the apparent imbalance between cell proliferation and death.

The immunocytochemical analysis of Bcl-2 indicated that its expression was restricted in the invaded stroma and down-regulated in moderately differentiated carcinoma. Bcl-2 immunoreactivity was higher in mild dysplasia than in severe dysplasia or invasive carcinoma. This suggests that apoptosis may be increased in cervical carcinoma as the anti-apoptotic protein expression level is reduced. Only limited studies have shown an apparent increase in Bcl-2 expression from mild dysplasia to invasive cancer, which proposes a strong linkage between Bcl-2 expression and different stages of cervical cancer (Grace *et al.*, 2003).

Recent studies indicated that p53 inactivation results from its association with viral oncoprotein E6. P53 inhibits tumour formation by avoiding proliferation in cells with damaged DNA, thus inhibiting cell growth until such DNA is repaired or by causing cells

to undergo apoptosis. P53 occasionally elicits Bax expression and inhibiting that of Bcl-2, thereby increasing rates of apoptosis. This supports a hypothesis that HPV infection not only plays a role in the aetiology of squamous cell carcinoma but might also be associated with the increased incidence of apoptosis in cervical carcinogenesis through regulation of the expression of p53 and Bcl-2 family (Kokawa *et al.*, 1999). A previous study indicated a significant correlation between the presence of HPV E6 protein and Bcl-2 (Grace *et al.*, 2003). This study suggests that HPV might be suppressing DWNN expression at the sites where Bcl-2 was highly expressed, or it might be suppressing Bcl-2 at the area where DWNN was highly expressed.

4.6 LightCycler RT-PCR

The ability to detect and quantify mRNA transcripts is a powerful tool for studying gene expression and regulation. To date, a few reports have described the application of LightCycler PCR to the detection, identification or quantification of genes. The DWNN gene was found to be highly expressed in tumour sections using *in situ* hybridization; this was confirmed by high amplification for the 5'1.1 kb mRNA. This indicates that the 5'1.1 kb mRNA is concentrated in the tumour. The exon 16 was also concentrated in contrast to the 3'6.1 kb mRNA, which means that the unspliced transcript was more highly expressed than the spliced transcript. Melting curve analysis showed a single peak which means specificity of amplification.

4.7 DWNN partial cDNAs.

NCBI Blast search revealed three DWNN partial cDNA's; RBBP6, P2P-R and PACT and a DWNN homologue Mpe 1 found in yeast. Mpe 1, an evolutionary conserved zinc-knuckle protein which takes part in RNA processing. Yeast Mpe 1 protein, is associated with zinc, RING finger domain (Vo *et al.*, 2001; Sakai *et al.*, 1995). DWNN-13kDa has the same sequence homology as Mpe 1. Because Mpe 1 is involved in the cleavage and polyadenylation of mRNA (Vo *et al.*, 1995), DWNN is thought to be involved in mRNA processing and this is also confirmed by its cytoplasmic localization. P2P-R is a nuclear protein and is associated to Ring finger and Zinc finger domains, proline rich; it also interacts with p53 and Rb tumour suppressor proteins (Scott *et al.*, 2003). Its cDNA encodes protein domains that take part in growth regulation. The binding of P2P-R to Rb, signifies localization sites of RNA processing in the nucleus. P2P-R gene products play a vital role in many biological and pathological processes, including apoptosis; its over-expression promotes apoptosis (Witte and Robert, 1997; Gao *et al.*, 2002). DWNN was found to be alternatively spliced at exon 16 and the difference between the two DWNN forms was 34 amino acids. Sakai and colleagues (1995) documented that RBBP6 is also alternatively spliced and the difference between the two RBBP6 forms was 34 amino acids. They also found that RBBP6 associates with two suppressor proteins p53 and Rb. The two alternatively spliced fragments of RBBP6 were aligned to that of DWNN-200 kDa. The 34 amino acid fragment was also identified in alternatively spliced RBBP6 in mice (Simons *et al.*, 1997). PACT also has the ability to bind p53 and Rb proteins (Simons *et al.*, 1997). P2P-R, PACT and RBBP6 are partial cDNA's of DWNN-200 kDa. They all bind both p53 and Rb tumour suppressor proteins, this illustrates their role in

programmed cell death and they all localise in the nucleus. Since DWNN complexes with both p53 and Rb tumour suppressor proteins, this supports the conclusion that DWNN is involved in apoptosis. This argument is based on the fact that the DWNN downstream sequence, RBBP6, also binds p53 and Rb proteins. From the correlation between elevated levels of DWNN and high apoptosis levels, one can argue that DWNN regulates the apoptosis cascade.

4.8 DWNN as a ubiquitin ligase

Tumourigenic traits of cancer cells are distinguished by deregulated protein synthesis involved in different cellular pathways including those of growth control, programmed cell death, signalling, and differentiation. Furthermore, changes in continuous degradation of proteins from these processes become a growing concept in carcinogenesis. Intracellular protein degradation is a highly selective and tightly regulated process (Sadat *et al.*, 2004). Proteolysis is significant for many cellular processes including apoptosis, MHC class 1 antigen presentation, cell cycle and intracellular signalling. Proteolysis is regulated by ubiquitin proteasome ligases (E1, E2 and E3). The three classes of E3 proteins include HECT, RING finger and Ubox domain types. The HECT domain family proteins directly catalyze the final attachment of ubiquitin to substrate proteins, in contrast, RING finger and Ubox E3's function as facilitator of interaction between E2 and target protein (Robinson and Ardley, 2004).

DWNN-13 kDa has a ubiquitin-like structure so it might be involved in ubiquitination reactions, but DWNN-200 kDa might be a ubiquitin ligase because of the RING finger domain on its downstream. Otherwise, further studies are being conducted to confirm this

prediction. Proteins with RING finger domain are known to be involved in the ubiquitination proteasome pathways, in which they serve as ubiquitin ligases. Ubiquitin ligases are also involved in the apoptotic cascade mechanism, example MDM2. The E3 ubiquitin ligase activity of MDM2 relies on its RING finger domain. The N-terminus of MDM2 binds to p53 and thus targets it for degradation. Its binding also hides the transactivation domain of p53, thereby suppressing its transcriptional activity (Fang *et al.*, 2000).

Cellular cervical transformation results from p53 inactivation by viral oncogene E6. This occurs by degradation of p53 by E6-AP ubiquitin ligase. E6-AP functions as E3 ligase in the ubiquitination of p53. MDM2 protein does not interfere with E6-AP mediated ubiquitination of p53 (Huibregtse and Beaudenon, 1996). DWNN-200 kDa protein potentially functions as p53 ubiquitin ligases. It may form part of E3 ubiquitin ligase and play a role in regulating levels of p53 and Rb due to the presence of the RING finger domain, thus regulating apoptosis. DWNN might be competing for the binding site to p53 with E6-AP or MDM2.

Yamasaki and Pagano (2004) reported that uncontrolled ubiquitin ligases targeting cell cycle regulatory proteins give rise to carcinogenesis. This was attained either by the elevated levels of ubiquitin ligases whose substrates are negative regulators of cell proliferation, or transformation of ubiquitin ligases targeting tumour suppressor proteins. Recent studies found that there are inhibitors that bind MDM2 in the p53 binding pocket; in so doing they inhibit the access of p53, resulting in its accumulation.